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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, A01H 4/00 — A1

(11) International Publication Number: WO 96/25504

(43) International Publication Date: 22 August 1996 (22.08.96)

(21) International Application Number: PCT/EP96/00712

(22) International Filing Date: 16 February 1996 (16.02.96)

(30) Priority Data:
9503099.5 17 February 1995 (17.02.95) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: GENETIC MODIFICATION OF PLANTS

(57) Abstract

A process for producing genetically modified Eucalyptus plant material comprising one or more stably incorporated DNA sequences of interest, which process comprises subjecting *Eucalyptus* cells or tissue to *Agrobacterium* mediated transfer of the DNA sequence(s) of interest, inducing shoot formation in transformed cells or tissue, and selecting transformed plant material, the induction of shoot formation being carried out in the presence of N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea and selection of transformed shoots being carried out, for example, in the presence of geneticin (G-418). The process is particularly useful for the transformation of clonal material derived directly or indirectly from a mature *Eucalyptus* tree.

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# GENETIC MODIFICATION OF PLANTS

This invention relates to the genetic modification of Eucalyptus, primarily for the commercial production of wood and wood products.

At present, commercial-scale planting of Eucalyptus forests uses planting stock which is produced either directly from seed or from rooted cuttings. In both of these production systems, traditional plant-breeding techniques are used to produce superior planting stock, with the potential for increased productivity of wood with desirable properties. Recent advances in plant genetic engineering have now made it possible stably to incorporate heterologous or homologous DNA into plants. These techniques therefore have the potential for the genetic improvement of Eucalyptus planting stock over and above those improvements obtainable using traditional breeding techniques.

The overall efficiency of genetic modification of plants is a function of the efficiency of the stable introduction of the heterologous or homologous DNA into cells, which is itself dependent on cell type and the method of transformation used, the efficiency of enrichment of transformed cells and tissues and the efficiency of the subsequent regeneration of viable plants from transformed cells. The specific strategies and methods that may be employed to achieve genetic modification are influenced by the biological properties and attributes of the plant involved. For example, during the development of transformation methods for any particular plant, the method of DNA delivery is preferably chosen so that DNA is introduced efficiently into cells that are totipotent and hence

have the capacity for subsequent regeneration into viable plants. However, the methods developed may not be relevant to other plants because, for example, the efficiency of stable introduction of DNA and/or the totipotency of the target cells and/or and the tissue culture techniques required to express that totipotentency may be different.

Some of these effects have a direct bearing on the development of methods for genetic modification of Eucalyptus species. For example, a method for the transformation of poplar shoot cultures using Agrobacterium tumefaciens, and the subsequent regeneration of the transformed cells is not satisfactory when Eucalyptus is used instead of poplar. As regards Eucalyptus itself, it has been reported that the introduction of heterologous DNA into seedling tissues of several Eucalyptus species including E. globulus using Agrobacterium rhizogenes or tumefaciens, is dependent on age. The greater level of tissue differentiation and an increased ability to produce polyphenolics are thought to be responsible for the reduction in susceptibility to genetic modification observed in older seedlings. Furthermore, seedlings over six weeks old were found to be incapable of forming adventitious shoots, tissues and cells, i.e. to undergo regeneration, unlike their younger counterparts.

There are several reports of attempts to produce genetically-modified Eucalyptus plants using methods which introduce DNA directly into cells, including electroporation of DNA into protoplasts, bombarding embryos with DNA-coated particles, and polyethylene-glycol mediated gene delivery, but success was achieved in some cases only, and in other cases no viable plants were recovered.

Many of the attempts to modify Eucalyptus genetically have used starting material that exhibits juvenile characteristics, in particular, seeds or seedlings, for example, young seedlings. Although sources of starting material having juvenile characteristics may be obtained by subjecting a mature tree to physical damage, chemical agents or a change in environmental conditions in order to induce the production of juvenile shoots, the cells and tissues of such shoots have different properties from those of young seedlings and are often less susceptible to transformation by Agrobacterium and/or are more recalcitrant to regeneration.

Some success has been achieved using Agrobacterium mediated delivery of heterologous genes into E. grandis and E. camaldulensis seedling material. One method involved the use of two different Agrobacterium strains to produce shooty callus from E. grandis seedlings; genetically-modified E. grandis shoots were subsequently produced from this callus. However, no success has yet been reported for genetic manipulation of seedlings of E. globulus, E. nitens or E. dunnii.

The exploitation of genetically manipulated Eucalyptus trees produced from the introduction of heterologous genes into tissues and cells derived from seedlings is much less commercially attractive than the exploitation of trees derived from genetically manipulated clonal material. Typically, the properties of seedlings are unknown as they have not been field-trialled. Introduction of heterologous gene(s) into cells or tissues derived from a seedling may therefore give rise to a mature tree that has undesirable properties and is not suitable for commercial exploitation. Field-trialling is therefore necessary after genetic manipulation, rather than

before, as would be the case of genetic manipulation of clonal material of established commercial value.

Field trials of juvenile genetically modified Eucalyptus plants involve a number of disadvantages. For example, Eucalyptus trees having superior properties cannot be selected on phenotype until marked changes in the Eucalyptus morphology and physiology have occurred. These changes, from a juvenile to a mature state, can take up to 3-4 years to materialise. The mature state is characterised by the ability of the Eucalyptus to produce flower buds and seeds. In order to select trees having superior wood properties, not only must the juvenile/mature phase-change have occurred, but also a sufficient amount of mature wood must be produced. This results in extended field-trialling periods being necessary. Furthermore, the resulting progeny of any sexual crosses may also have to be field-trialled after each step of the breeding programme. These factors all extend the time taken for commercial exploitation of genetically modified Eucalyptus trees produced from juvenile starting material, for example, from seedlings.

In practice, to maximise commercial yields and improve product quality, it is desirable to establish clonal Eucalyptus plantations, that is to say, plantations of genetically identical trees, that are selected on the basis of one or a number of superior phenotypic properties after the identification of those properties in an individual within a population of trees. The population may be a natural population or a superior population derived by traditional breeding techniques or by genetic modification. Suitable material for clonal propagation can be removed from selected tree(s) having superior properties. The process of clonal propagation is repeated serially

until there is sufficient cloned material to enable a plantation to be established.

Accordingly, trees that have been field-trialled and identified as having superior properties are the product of a significant investment of effort in breeding and selection. Such trees therefore represent a valuable asset that can be exploited rapidly without the need for further plant-breeding steps as the trees can be propagated directly by the rooting of cuttings or by the techniques of micropropagation, and used as the planting stock for the establishment of commercial forests. The ability to introduce heterologous and/or homologous gene(s) into the cells and tissues of selected field-trialled trees would therefore allow additional commercially significant properties to be introduced into those already valuable trees and hence facilitate the commercial exploitation of the resulting trees in clonal Eucalyptus forests.

However, in spite of the commercial desirability of genetically modifying clonal material from selected trees having superior properties, there has been notable lack of success, the clonal material from mature trees proving recalcitrant both to genetic modification and/or to regeneration.

It has been found in practice that methods suitable for Agrobacterium mediated transformation of seedlings are not directly applicable to genetic manipulation of clonal material. For example, genetically-modified E. grandis seedlings were produced using an Agrobacterium mediated transformation system and some of the resultant genetically-modified plants were field--trialled (Edwards GA (1993) Oral Presentation, Tree Biotechnology Group Meeting, Coventry, England and Department of the Environment of the UK, Public Register of H.M. Pollution Inspectorate (1993)).

However, when the methods that were used successfully for the production of genetically-modified E. grandis seedlings were applied to a large number of clones of E. grandis, no genetically modified plants were recovered.

It cannot be predicted that methods that are successful for regeneration of micropropagated material will be applicable to regeneration of genetically-modified clonal material. For example, Laine & David (Plant Cell Reports 13:473-476 (1994)) report successful regeneration of E. grandis clones from callus-tissue initiated from leaves of in vitro micropropagated shoots that had previously been field-trialled, using a particular tissue culture medium and state that the medium could be used to promote regeneration in genetically-modified E. grandis clones. However, our work (which incorporated this medium into a method used to regenerate over 5,000 E. grandis leaf explants that had been previously co-cultivated with an Agrobacterium using a method that had previously been used successfully to produce genetically-modified seedlings) failed to substantiate this, despite using the same E. grandis clones as Laine & David. The leaves showed an abnormal response, in that the cells around the cut edge produced phenolic compounds, and many subsequently died. This is consistent with observations made by others. Callus which contained the heterologous gene(s) was rarely produced, and the frequency of regeneration was too low to give rise to genetically-modified shoots. Hence the regeneration protocol is not compatible with Agrobacterium mediated transformation of E. grandis explants derived from field-grown plants (clones).

Such examples serve to demonstrate that there is no reasonable expectation that methods that can be used

successfully in the genetic modification of Eucalyptus cells and tissues derived from seedlings can be applied successfully to cells and tissues derived from clonal material, particularly the advantageous clonal material obtained from mature trees, especially field-trialled trees having superior properties.

The present invention provides a process for producing genetically modified Eucalyptus plant material comprising one or more stably incorporated DNA sequences of interest, which comprises subjecting Eucalyptus cells or tissue to Agrobacterium mediated transfer of the DNA sequence(s) of interest, inducing shoot formation in the resulting transformed cells or tissue, and selecting the transformed material, the induction of shoot formation being carried out in the presence of N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea. The NPTII gene is preferably used as a selective marker gene, and selection of the transformed material may be carried out using G418, which is also known as geneticin, or neomycin.

The resulting selected transformed plant material, for example, transformed callus, regenerating shoots or regenerated shoots, may be grown into plants directly or may be propagated vegetatively, especially by micropropagation, to increase stock before being grown into plants. Resulting genetically modified plants may themselves be cloned, for example, by micro-propagation, or by any other method of vegetative propagation, for example, by cuttings.

By the process of the present invention, cells and tissue derived via vegetative propagation i.e. clonal material, especially clonal material from mature Eucalyptus trees exhibiting superior phenotypic properties, for example, E. grandis, E. saligna, E. dunnii or E. camaldulensis trees or trees that are

hybrids thereof, may be modified genetically and regenerated into viable plants. The process of the present invention may also be applied to cells and tissue derived from Eucalyptus seedlings, and enables the production of viable, genetically modified plants from seedlings of species of Eucalyptus that have previously proved recalcitrant to genetic manipulation, for example, E. globulus, E. nitens and E. dunnii. Mature Eucalyptus trees obtained from plant material modified genetically according to the present invention and products obtained from such trees, for example, timber, wood pulp and fuel wood, are valuable commercial commodities.

In the process of the invention, cells or tissue are cultured, in a suitable medium, with Agrobacterium cells capable of delivering one or more DNA sequence(s) of interest that are functional in plants, and that can be transferred to the cells or tissue. A DNA sequence of interest may be heterologous to the Eucalyptus or homologous. Many examples of suitable DNA sequences are known. For example, the DNA may function to impart to the Eucalyptus a phenotypic property, e.g. resistance to a herbicide such as glyphosate, to modify wood fibre quality or the chemical components of wood, to modify rooting ability of vegetative propagules, for example, cuttings, to modify tree architecture and branching, or to induce sterility.

Suitable media and conditions for plant culture are known. Optimal media and culture conditions for any particular starting material may be determined by routine methods if they are not already known. For the process of the present invention, the medium may contain glutamate and/or ascorbic acid, in order to promote regeneration of shoots at high efficiency. The starting pH may be 5.0-5.6. The induction of shoot

formation and the selection of transformed material according to the present invention are preferably carried out by culture on a solid medium or using another static culture medium. Examples of media suitable for use in the process of the present invention for inducing shoot formation, for selection of transformed cells and tissue and for multiplication and inducing root formation of genetically modified Eucalyptus are given herein.

The substituted phenylurea N-(2-chloro-4-pyridyl)-N'- phenylurea, often known as 4-PU or CPPU, is incorporated into the culture medium used for shoot induction and preferably also in the medium used for selection of transformed shoots, and also for shoot regeneration, either as the sole cytokinin or with other cytokines. CPPU induces bud formation in Eucalyptus at high frequency and, unlike some other phytohormones and plant growth factors, a further effect is that the buds produced are capable of further development into shoots. Other substituted phenylureas may be used instead of or in addition to CPPU provided they are capable of inducing, at high frequency, the formation of buds that are capable of further development. The suitability of any particular substituted phenylurea for any particular Eucalyptus starting material and appropriate concentrations of the selected phenylurea and regimes for its use may be determined by routine methods.

By conventional methods, successful transformation of cells or tissue, for example, shoots, is generally determined using any suitable characteristic as a marker. In the present invention, the NPTII gene may be used as the marker gene and the resistance to a phytotoxic agent conferred by that gene, for example, resistance to G-418 (also known as geneticin) or to

neomycin may be used as the characteristic for selection of transformed cells or tissue. Any other DNA sequence that confers the same or similar resistance may be used as the selectable marker. The selective agent should be used in a concentration and in a regime that enables selection of the transformed material, for example, transformed callus, transforming shoots and transformed shoots.

The cells or tissue used as starting material for genetic modification according to the present invention may be derived from seedlings, especially young seedlings. The process of the present invention is particularly useful for the genetic modification of cells and tissue obtained from E. globulus, E. nitens and E. dunnii seedlings, as no such process has been disclosed previously.

As set out above, there are potential advantages in genetically modifying clonal material, for example, cell or tissue clonal material that is vegetatively derived, directly or indirectly, from vegetative tissues of trees, especially mature trees, that have been selected, or are selectable, for favourable characteristics. The cell or tissue material may be obtained directly from a plant grown in the field or a greenhouse; it may be used in non-sterile form, i.e. without the use of an intervening micropropagation step, for the introduction of heterologous (or homologous) gene(s). Alter-natively, the cells or tissue may be derived indirectly from selected trees, that is to say, the cells or tissue taken from the selected tree is subjected to micropropagation before genetic manipulation. The tissue may be leaves, stems or petioles.

In the case of clonal material, the starting material is preferably obtained from E. grandis, E.

dunnii, E. saligna or E. camaldulensis, or from a variety, cultivar or hybrid thereof.

Any suitable Agrobacterium vector may be used to mediate genetic modification of the Eucalyptus material. The Agrobacterium tumefaciens strain used to transform E. globulus and E. nitens seedlings, E. grandis clones and E. grandis/E. camaldulensis hybrid clones as described in the Examples is the disarmed strain EHA101A containing the binary Ti plasmid pSCV1.6. That strain may be used for the transformation of other Eucalyptus. Suitable binary Agrobacterium-Ti plasmid vector systems have been fully described elsewhere, e.g. in EP-A-0120516.

Figure 1 of the accompanying drawings is a map of a plasmid identified herein as pSCV1, which is used in the production of plasmid pSCV1.6. Figure 2 is a map showing the T-DNA of a plasmid identified herein as pSCV1.6, which may be used in the process of the present invention.

In Figure 1 $Amp^R$ and $Gm/Km^R$ denote antibiotic resistance genes for plasmid selection in bacteria. trfA, trfB, RK2 and Col E1 origins denote baterial replication functions. OD denotes an overdrive (T-DNA transfer enhancer) sequence. Bam H1, Bcl 1, Cla 1 etc denote restriction endonuclease recognition sequences. Map units are given in Kilo base pairs of nucleotide sequence.

In Figure 2 the orientation of the genes and the region of DNA for transfer to plants are shown. The abbreviations given in Figure 2 have the following meanings: B = Bam H1; Bg = Bgl II; C = Cla 1; E = Eco R1; EV = Eco RV; H = Hind III; K = Kpn 1; P = Pst 1; S = Sac 1; Sm = Sma 1; Sp = Sph 1; X = Xba 1; Xh = Xho 1; OD = Over-drive (T-DNA transfer enhancer)

The present invention is readily adaptable to the production of a single clone, or of a variety of clones. A mono-clonal or multi-clonal forest can thus be grown, based on this invention. Improved yields and better quality product can be and are obtained from forests grown from seed, but the overall gains are typically smaller when compared to those achieved in forests planted with genetically identical trees (clones) derived from a single superior tree.

Hence, clones that have been grown in the field are the optimal source of starting material for further genetic improvement, by inserting additional genes using the techniques of genetic modification according to the present invention. Mature trees grown from genetically modified plant material obtained according to the present invention and products obtained from such trees are valuable commercial commodities.

The following non-limiting Examples illustrate the invention.

EXAMPLES

EXAMPLE 1

TRANSFORMATION OF E. GLOBULUS AND E. NITENS SEEDLINGS

A. Agrobacterium strain, binary Ti plasmid vector and gene construct

a) Disarmed Agrobacterium strain

The construction of A. tumefaciens strain EHA101 has been described by Hood et al., 1986. The strain consists of a derivative of the of nopaline A. tumefaciens strain C58 in which the native Ti plasmid has been removed and replaced with the disarmed Ti plasmid pEHA101 in which the wild-type T-DNA (ie opine synthesis and phytohormone genes) has been deleted from the Ti plasmid and replaced with a bacterially-xpressed kanamycin/neomycin resistance gene. The disarmed plasmid pEHA101 is a derivative of the wild-type Ti

plasmid pTiBo542 isolated from A. tumefaciens strain Bo542 (AT4) which is a L,L-succinamopine producing strain (Hood et al., 1986). Strain EHA101A is a chloramphenicol resistant mutant of strain EHA101 which was isolated by Olszwelski et al., 1988.

b) Binary vector construct

The strain used in the transformation also contains the binary Ti plasmid pSCV1.6, which is a derivative of pSCV1. Genetic manipulations involving these plasmids were performed using standard techniques (Sambrook et al., 1989).

The component parts of pSCV1 are derived from the following (gram-negative) plasmids: the sequence used for the right DNA border and overdrive sequence was synthesised using sequence information from from the TL right border of the octopine Ti plasmid pTiA6 (Peralta et al., 1986). The left border was synthesised using sequence information from the TL of the same Ti-plasmid (Simpson et al., 1982) and is identical to the TL left border of the octopine plasmid pTiACH5 (Holsters et al., 1983). Octopine-type border sequences were used as these have been shown to promote more efficient tumour formation when used in conjunction with the hypervirulent strain EHA101 (Hood et al., 1986). The 97bp polylinker containing restriction enzyme sites for cloning genes into the T-DNA was derived from pUC19 (Yannish-Perron et al., 1985). The high copy number origin of replication which is active in E. coli cells but not Agrobacterium cells was derived from pUC19 (Yannish-Perron et al., 1985). The origin of replication of pUC 19 which was itself originally derived from the plasmid ColE1, a plasmid isolated from E. coli. The actual pUC sequence used has been extensively deleted to remove some non-functional (superfluous) DNA sequences. The low copy number

origin of replication which is active in both E. coli cells and Agrobacterium cells was derived from the the broad host-range Inc P plasmid RK2. The origin used is a minimal 4.3kb origin which was constructed by deleting most of the non-functional sequences originally present in the wild-type RK2 plasmid (Thomas et al., 1980). The minimal origin therefore contains only two genes (trf A and trf B) and associated non-coding sequences needed for replication in bacteria. The bacterially-expressed gentamicin/kanamycin resistance gene was derived from the plasmid pSa (Edwards, 1988) and is probably an aminoglycoside acetylase (Valantine and Kato, 1989). It has no apparent homology to the neomycin phosphotransferase II coding region (Edwards, 1988). The bacterially-expressed ampicillin/carbenicillin resistance (ß-lactamase, bla) gene was cloned from pUC19 (Yannish-Perron et al., 1985). A genetic and restriction map of pSCV1 is shown in Figure 1.

In Figure 1 $Amp^R$ and $Gm/Km^R$ denote antibiotic resistance genes for plasmid selection in bacteria. trfA, trfB, RK2 and Col E1 origins denote baterial replication functions. OD denotes an overdrive (T-DNA transfer enhancer) sequence. Bam H1, Bcl 1, Cla 1 etc denote restriction endonuclease recognition sequences. Map units are given in Kilo base pairs of nucleotide sequence.

pSCV1.6 is a derivative of pSCV1, into which a plantexpressed ß-glucuronidase (GUS) gene and a plant-expressed kanamycin resistance gene were cloned between the T-DNA borders. The CaMV-NPTII was derived from the construct of Fromm et al., 1986. However, it has been reported that several of the most common NPTII genes used in plant genetic-manipulation encode a mutant enzyme that has a reduced ability to detoxify

kanamycin (Yenofsky et al., 1990). The mutation involves a single base change, resulting in the replacement of a glutamic acid residue by an aspartic acid at the active site of the neomycin phospho-transferase (NPTII) enzyme (originally isolated from the bacterial transposon Tn5). While the stability of the mRNA and the protein appeared unaffected by the mutation, the enzyme activity towards kanamycin is significantly reduced. The presence of the mutation in a gene can be identified by checking for the loss of a site for the restriction endonuclease XhoII in the NPTII coding sequence. This mutation was found to be present in the CaMV-NPTII gene of Fromm et al., 1986 and was repaired in the following manner. The plasmid pSUP2021 (Simon et al, 1983) is approximately 10kb in size and includes a complete copy of the transposon Tn5. Digestion of this plasmid with Pst 1 and Sma 1 gives a 788bp fragment that extends from position 1730 to 2518 within Tn5 (Beck et al., 1982). This fragment was isolated and restricted with Sph 1 (giving fragments of 352 and 436 bp) or XhoII (giving fragments of 120, 246, 394 and 28 bp), and is therefore "wild-type" with respect to the mutation at position 2096. The Pst 1/Sma 1 fragment was subcloned into Pst 1/Sma 1 cut pUC19 to give pTn5sub. This was then digested with Sma 1 and ligated with 8mer phosphorylated Bam H1 linkers. A clone in which the Sma 1 site had been converted to a Bam H1 site (pTn5subA) was then digested with Sph 1 and Bam H1 and the 436bp fragment (from position 2082 to 2518) isolated. This was used in a tripartite ligation with the 542 bp Bam H1/Sph 1 fragment from pCaMVNeo (positions 1540 to 2082) and Bam H1 digested pUC19. Recombinant's were restricted with Bam H1 and Sph 1 to ensure that they contained both the 436 and 542 Bam

H1/Sph 1 fragments, and Xho II to confirm that the site at position 2096 had been restored. This construct has a Bam H1 fragment which contains the NPTII gene coding sequence which is essentially identical to the Bam H1 fragment used by Fromm et al., (1986) to make pCaMVNeo, except that the mutation has been corrected. This construct was designated pneoNeo. The Bam H1 insert of pneoNeo containing the NPT11 coding sequence was then isolated and religated with the large (approx. 3 kb) fragment isolated from Bam H1 restricted pCaMVNeo, this fragment containing the vector plus CaMV promoter and nopaline synthase gene 3' termination sequence. Recombinants were checked against pCaMVNeo for the correct orientation using both Pvu II (2 sites) or Eco R1/Sph 1 (both unique), giving pCaMVneoNeo. This was again checked for the correct number of Xho II sites.

The Hind III fragment from pCaMVneoNeo containing the restored plant-expressed kanamycin resistance gene was cloned into the Hind III site of pSCV1 to give the plasmid pSCV1.2. pSCV1.2 was partially digested with HindIII and the linear 10.2kb product isolated. This was dephosphorylated with calf intestinal alkaline phosphatase and ligated with a 2.8kb Hind III DNA fragment containing a plant expressed ß-glucuronidase gene (CaMV-GUS INT gene) isolated from the plasmid pGUS INT which has been described by Vancanneyt et al., 1990.

A map of the T-DNA in the resultant construct (pSCV1.6), indicating the orientation of the genes and the region of DNA for transfer to plants are shown in Figure 2.

In Figure 2 the abbreviations given in the map have the following meanings: B = Bam H1; Bg = Bgl II; C = Cla 1; E = Eco R1; EV = Eco RV; H = Hind III; K = Kpn 1; P = Pst 1; S = Sac 1; Sm = Sma 1; Sp = Sph 1; X =

Xba 1; Xh = Xho 1; OD = Over-drive (T-DNA transfer enhancer)

c). Introduction of the binary plasmid vector pSCV1.6 into the disarmed A. tumefaciens strain

Cells of Agrobacterium tumefaciens strain EHA101A were transformed by electroporation using a Biorad Gene Pulser as described by Wen-jun and Forde (1989).

B. Transformation of E. globulus and E. nitens seedlings

a) Plant material

Seedlots of E. globulus collected from Curanilahue, Chile were obtained from Forestal Y Agricola Monte Aguila, Nacimiento, Chile. Seeds of E. nitens provenance Errinundra S.F. were obtained from CSIRO Division of Forestry, Australian Tree Seed Centre, Queen Victoria Terrace, Canberra, Australia (supplier's reference number 16341).

b). Seed germination and preparation of explants for transformation

Seeds of E. globulus were surface sterilised in a solution of 15% v/v Milton solution (Proctor & Gamble Limited, Egham, Surrey, UK) containing 0.1% Tween 20 for 30 minutes with gentle agitation followed by three ten minute rinses in sterile double distilled water. Seeds of E. nitens were surface sterilised in a solution of 25% v/v Milton solution containing 0.1% Tween 20 for 10 minutes with gentle agitation followed by three ten minute rinses in sterile double distilled water. Sterilised E. globulus seeds were sown on a plant culture medium consisting of half-strength macro and micro elements as described by Murashige and Skoog (1962), vitamins as described by Morel and Wetmore (1951), 20 g $l^{-1}$ sucrose and 2 g $l^{-1}$ phytagel (Sigma), pH adjusted to 5.8 with KOH. The seedlings were

germinated and grown in a controlled environment growth room at a temperature of 23°C and a 16 hour photoperiod with a light intensity of 40μmol $m^{-2}s^{-1}$. Sterilised E. nitens seedlings were sown and germinated on a plant culture medium and under the conditions described for E. globulus except that germination was conducted at 16° C prior to the transfer to the 23°C growth room for subsequent growth of the seedlings as described for E. globulus.

Hypocotyls were prepared from 8-26 day-old E. globulus and E. nitens seedlings and explants 2.5-5mm long were excised from the apical end (upper one third) of the hypocotyl. The hypocotyl explants were transferred to liquid seedling shoot induction medium (see later) until required. The liquid medium contains the same constituents as the solid medium except that the phytagel is omitted.

c) Preparation of Agrobacterium inoculum

Overnight liquid cultures of Agrobacterium tumefaciens strain EHA101A containing the binary plasmid pSCV1.6 were grown on YEB medium (tryptone 5 g $l^{-1}$, yeast extract 1 g $l^{-1}$, beef extract 5 g $l^{-1}$, magnesium sulphate 0.46 g $l^{-1}$, pH 7.2 and sucrose 5 g $l^{-1}$ added after autoclaving) containing 50 mg $l^{-1}$ chloramphenicol, 25 mg $l^{-1}$ neomycin and 15 mg $l^{-1}$ gentamicin at 28°C with vigorous shaking. 10 μl of a fresh overnight liquid culture was inoculated into 25ml of fresh media and grown for 24 h. The cells were harvested by centrifugation at 6000g for 10 minutes, resuspended in 2mM $MgSO_4$ and repelletted. The cells were washed once more in 2mM $MgSO_4$ and once in liquid clone co-cultivation medium (see later). The cells were finally resuspended in liquid clone co-cultivation medium and diluted to a density of $10^9$ cells $ml^{-1}$ ready for co-cultivation with the explants.

d). Inoculation of explants with Agrobacterium and regeneration of putative transgenic shoots

Hypocotyl explants were incubated with the Agrobacterium suspension, prepared as described above, for 15 minutes in a sterile 9 cm petri dish. The dish was placed on an orbital shaker and shaken gently at 23° C during the incubation. After incubation, excess bacterial suspension was removed from the explants by blotting with filter papers and the hypocotyl explants were transferred to solid seedling shoot induction medium (half-strength macroelements and iron as described by Murashige and Skoog (1962), microelements as described by Bourgin and Nitsch (1967), vitamins as described by Nitsch and Nitsch (1965), 30 g $l^{-1}$ sucrose, 1 mg $l^{-1}$ CPPU (N-(2-chloro-4-pyridyl)-N'-phenylurea), 0.1 mg $l^{-1}$ NAA (naphthaleneacetic acid), pH adjusted to 5.6 with KOH) solidified with 3 g $l^{-1}$ phytagel (Sigma). The inoculated hypocotyls were incubated for 48 hours under the same conditions used for the germination of E. globulus seeds. The hypocotyl segments were then washed twice (3 hours per wash) in liquid regeneration medium containing 400 mg $l^{-1}$ augmentin (Beechams; SKB) at 23°C with gentle shaking. Excess liquid was then removed by blotting the explants with filter paper and the explants transferred to solid seedling shoot induction medium containing 300 mg $l^{-1}$ augmentin and 10 mg $l^{-1}$ G-418 (geneticin). The hypocotyls were incubated for 4 weeks (with one subculture onto fresh medium after 2 weeks) under the same conditions used for the germination of E. globulus seeds. The explants were then subcultured onto seedling regeneration medium No. 1 (half strength macroelements and iron as described by Murashige and Skoog (1962), microelements as described by Bourgin and Nitsch (1967), vitamins as described by Nitsch and

Nitsch (1965), 30 g $l^{-1}$ sucrose. 0.5 mg $l^{-1}$ BAP (6-benzylaminopurine) 0.1 mg $l^{-1}$ NAA, 50 mg $l^{-1}$ arginine, 50 mg $l^{-1}$ serine, 50 mg $l^{-1}$ glycine, 500 mg $l^{-1}$ glutamine, 50 mg $l^{-1}$ ascorbic acid, 300 mg $l^{-1}$ augmentin, 10 mg $l^{-1}$ G-418, pH adjusted to 5.6 with KOH, 3 g phytagel). The explants were incubated for two weeks at 23°C with a 16 hour illumination regime (40 µmol $m^{-2}s^{-1}$). Explants with regenerating callus were then subcultured onto seedling regeneration medium No. 2 (as seedling regeneration medium No. 1 except that the BAP concentration is 0.5 mg $l^{-1}$) and subcultured at intervals of two weeks using the same environmental conditions described for incubation on seedling regeneration medium No. 1.

e). Multiplication and rooting of putative genetically modified E. globulus and E. nitens shoots

Putative genetically modified shoots developing on the seedling regeneration medium containing G-418 were excised from the callus and transfered to E. globulus/E. nitens micropropagation medium (full strength macroelements, microelements and vitamins, as described by Murashige and Skoog (1962), 20 g $l^{-1}$ sucrose, 0.01 mg $l^{-1}$ indole-3-butyric acid (IBA), 0.1 mg $l^{-1}$ BAP, pH adjusted to 5.6 with KOH, 2 g $l^{-1}$ phytagel) containing 300 mg $l^{-1}$ augmentin and propagated at 23°C using 16 hour day illumination regime (50-70 µmol $m^{-2}s^{-1}$). The multiplying shoots were divided and subcultured onto fresh E. globulus/E. nitens micropropagation medium at 4 weekly intervals. Putative genetically modified shoots were rooted by removing shoots from the micropropagated cultures, removing any callus from the stems and transferring the shoots to E. globulus/E. nitens root induction medium (quarter-strength macroelements and microelements as described by Murashige and Skoog (1962), 20 g $l^{-1}$

sucrose, 40 mg $l^{-1}$ IBA, pH adjusted to 5.6 with KOH, 2 g $l^{-1}$ phytogel) and incubating the shoots for 24h under the environmental conditions used for micropropagation. Following the root-induction step, shoots were transferred and the stems inserted into a polypropylene fibre substrate (Milcaps, Milcap France, S.A., Chemin de Montbeau, 49340 Nuaille, France) soaked in liquid E. globulus/E. nitens rooting medium (quarter-strength macroelements and microelements as described by Murashige and Skoog (1962), 20 g $l^{-1}$ sucrose, pH adjusted to 5.6 with KOH) and incubated under the environmental conditions used for micropropagation and root induction. When actively growing roots were visible growing through the polypropylene plug, the plants were transferred to approximately 7.5cm (3 inch) square plant pot filled with coco-peat. The plants were placed inside a mist propagator and slowly hardened off by reducing the humidity over a period of a week. After three to four weeks, the plants were transferred to approximately 17.5cm (7 inch) pots and placed in a glasshouse facility. The plants were grown under natural daylight and were watered daily.

EXAMPLE 2

TRANSFORMATION OF E. GRANDIS CLONES, E. GRANDIS/E. CAMALDULENSIS HYBRID CLONES AND E. SALIGNA/E. TERETICORNIS HYBRID CLONES

a) Plant material

E. grandis clone 91/4 and E. grandis/E. camaldulensis hybrid clones 11/25 and 11/15 were supplied by the South African Forestry Research Institute, PO Box 727, Pretoria 0001, Republic of South Africa (now FORESTEK, Private Bag X11227, Nelspruit 1200, South Africa). E. dunnii clones G7 and G14 were obtained from Mondi Forests, NTE House, P.O. Box 39, Pietermaritzburg 3200, Republic of South Africa. E.

saligna/E. tereticornis hybrid 2.32 was obtained from Congolaise de Development Forestier, B.P 1227 Pointe Noire, Republique Du Congo. Stock plants were obtained by felling mature trees and harvesting cuttings from new growth arising from from epicormic buds in the stump. Cuttings were rooted using routine silvacultural techniques and subsequently potted into 10 litre pots and maintained in the glasshouse as hedged stockplants. Where required, in vitro micropropagated shoot cultures were initiated from these stockplants by harvesting nodal stem explants from stockplants and disinfecting by immersion in a 20% v/v Milton solution containing 0.1% v/v Tween 20 for 10 minutes with gentle agitation. The nodal stem explants were then briefly rinsed three times in sterile distilled water and cultured on shoot multiplication medium (190 mg $l^{-1}KNO_3$, 825 mg $l^{-1}$ $NH_4NO_3$, 220 mg $l^{-1}$ $CaCl_2.H_2O$, 925 mg $l^{-1}MgSO_4$, 85 mg $l^{-1}$ $KH_2PO_4$, half-strength Murashige and Skoog basal salt micronutrient solution (catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 10 g $l^{-1}$ sucrose, 0.04 mg $l^{-1}$ BAP, 300 mg $l^{-1}$ augmentin, pH adjusted to 5.6 with KOH, 2 g $l^{-1}$ phytagel). The cultures were propagated at 23°C using a 16 hour day illumination regime (50-70 µmol $m^{-2}$ $s^{-1}$). The multiplying shoots were divided and subcultured onto fresh clonal shoot multiplication medium at 4 weekly intervals.

b) Preparation of explants for transformation.

Leaf, petiole or stem explants from the clones were prepared directly from axenic micropropagated shoot cultures or rooted micropropagated shoots without disinfection (protocols for micropropagation and susequent rooting of shoots are given below). Alternatively, leaf, petiole or stem explants were prepared from ramets (either produced via

micropropagation or by cuttings) grown in the greenhouse or in the field and disinfected prior to co-cultivation with Agrobacterium tumefaciens. In this case, young scions with healthy leaves less than 3cm in length were harvested from the upper portion of the crown from vigorous plants of less than 1.5 metres in height, and disinfected by immersion in a 20% v/v Milton solution containing 0.1% v/v Tween 20 for 10 minutes with gentle agitation. The scions were then rinsed three times in sterile distilled water prior to dissection. 3-5mm diameter leaf explants or 2-4mm long sections of stem or petioles were prepared from the scions and placed in liquid clonal co-cultivation medium (see below) until required for co-cultivation with the A. tumefaciens strain.

c) Preparation of Agrobacterium tumefaciens inoculum

Agrobacterium tumefaciens strain EHA101A containing the binary plasmid pSCV1.6 was prepared for inoculation of the clonal explants as has been described in the Example 1 except that the final wash and subsequent resuspension of the cells was conducted in liquid clone co-cultivation medium (see below).

d) Inoculation of explants with Agrobacterium and regeneration of putative transgenic shoots.

Leaf, petiole or stem explants of the clones previously described were co-cultivated with the Agrobacterium suspension, prepared as described previously, for 15 minutes in a sterile 9 cm petri dish. The dish was placed on an orbital shaker and gently shaken at 23°C during the incubation. After incubation, excess bacterial suspension was removed from the explants by blotting with filter papers and the hypocotyl explants were transferred to solid clone co-cultivation medium (750 mg $l^{-1}$ $KNO_3$, 250mg $l^{-1}$ $MgSO_4.7H_2O$, 250 mg $l^{-1}$ $NH_4H_2PO_4$, 100mg $l^{-1}$ $CaCl_2.2H_2O$,

20 g $l^{-1}$ sucrose, 600 mg $l^{-1}$ 2-[N-morpholino]ethane-sulphonic acid (MES), half-strength Murashige and Skoog basal salt micronutrient solution (Sigma catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 0.1 to 1 (eg 1) mg $l^{-1}$ CPPU, 0.465 mg $l^{-1}$ NAA, pH adjusted to pH 5.5 with KOH, 3 g $l^{-1}$ phytagel). The explants were co-cultivated with the Agrobacterium strain for 48 h in the dark at 23°C. After incubation, excess bacterial suspension was removed from the explants by blotting with filter paper and the explants were then washed twice (3 hours per wash) in liquid clone co-cultivation medium containing 400 mg $l^{-1}$ augmentin at 23°C with gentle shaking. The explants were then transferred to clonal shoot induction medium (as for clone co-cultivation medium but containing 500 mg $l^{-1}$ glutamine, 50 mg $l^{-1}$ ascorbic acid, 300 mg $l^{-1}$ augmentin and 30 mg $l^{-1}$ G-418. The explants were incubated in the dark at 23°C for 4 weeks with subculture to fresh medium after 2 weeks and at the end of the period of incubation in the dark. The cultures were then transferred to continuous light (40 µmol $m^{-2}$ $s^{-1}$) and incubated at 23°C. The cultures were then subcultured every two weeks onto fresh clonal shoot induction medium until significant numbers of shoot primordia were visible. The explants were subcultured onto clonal shoot elongation medium (as clonal shoot induction medium) but with the CPPU ommitted, the NAA concentration adjusted to 0.112 mg $l^{-1}$ and containing 1.16 mg $l^{-1}$ BAP and incubated at 23°C under continuous light (40 µmol $m^{-2}s^{-1}$).

e) Multiplication and rooting of putative genetically modified shoots

Putative genetically modified shoots were excised from the cultures and transferred to clonal shoot multiplication medium (190 mg $l^{-1}$ $KNO_3$, 825 mg $l^{-1}$

$NH_4NO_3$, 220 mg $l^{-1}$ $CaCl_2.H_2O$, 925 mg $l^{-1}$ $MgSO_4$, 85 mg $l^{-1}$ $KH_2PO_4$, half-strength Murashige and Skoog basal salt micronutrient solution (catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 10 g $l^{-1}$ sucrose, 0.04 mg $l^{-1}$ BAP, 300 mg $l^{-1}$ augmentin, pH adjusted to 5.6 with KOH, 2 g $l^{-1}$ phytagel). The cultures were propagated at 23°C using a 16 hour day illumination regime (50-70 µmol $m^{-2}$ $s^{1)}$. The multiplying shoots were divided and subcultured onto fresh clonal shoot multiplication medium at 4 weekly intervals. Once rapidly growing shoot cultures had been established, individual shoots were transferred to rooting medium (as clonal shoot multiplication medium but with the BAP ommitted and containing 0.2 mg $l^{-1}$ IBA) and returned to the growth room. Shoots with developing roots were transferred to a sterile Jiffy-7 peat pellet (Jiffy Products (UK) Limited, 14/16 commercial Road, March, Cambridge, UK) in a Magenta pot (Sigma) for root establishment.

When actively growing roots were visible growing through the peat pellet, the plant was transferred to an approximately 7.5 cm (3 inch) square plant pot filled with coco-peat. The plants were placed inside a mist propagator and slowly hardened off by reducing the humidity over a period of a week. After three to four weeks, the plants were transferred to approximately 17.5 cm (7 inch) pots and placed in a glasshouse facility. The plants were grown under natural daylight and were watered daily.

EXAMPLE 3

TRANSFORMATION OF E. DUNNII CLONES AND SEEDLINGS

a) Plant material

E.dunnii seed was obtained from Compania Forestal Oriental, 18 de Julio 818, Paysandu 6000, Uruguay (seed batch reference no. 1278). E. dunnii clones G7 and G14

were obtained from Mondi Forests, NTE House, P.O. Box 39, Pietermaritzburg 3200, Republic of South Africa. Stockplants were obtained as described in Example 2.

b) Seed germination and preparation of explants for transformation

Seeds of E. dunnii were surface sterilised as previously described for E. nitens seeds in Example 1. Disinfected seeds were germinated as previously described for E. globulus in Example 1. Hypocotyl explants for prepared as previously described for E. globulus and E. nitens in Example 1. The hypocotyls were transferred to a reservoir of liquid clonal co-cultivation medium as described in Example 2. Explants from clones were prepared as described in Example 2.

c) Preparation of Agrobacterium tumefaciens inoculum

Agrobacterium tumefaciens strain EHA101A containing the binary plasmid pSCV1.6 was prepared for inoculation of the clonal explants as has been described in xample 1 except that the final wash and subsequent resuspension of the cells was conducted in liquid clone co-cultivation medium as described in Example 2.

d) Inoculation of explants with Agrobacterium and regeneration of putative transgenic shoots

Inoculation of E. dunnii seedling and clonal explants with Agrobacterium and regeneration of putative transgenic shoots was conducted as described in Example 2, except that selection for genetically modified shoots from seedlings was conducted on 10 mg $l^{-1}$ G-418 instead of 30 mg $l^{-1}$ G-418 used in Example 2.

e) Multiplication and rooting of putative genetically modified shoots

Multiplication and rooting of putative genetically modified shoots of E. dunnii obtained from either seedling or clonal explants was conducted as described for E. globulus and E. nitens in Example 1.

Note: In the above Examples, G418 may be used at a concentration of 10 to 40 mg $l^{-1}$.

EXAMPLE 4

BIOCHEMICAL AND GENETIC ANALYSIS OF GENETICALLY MODIFIED EUCALYPTUS PLANTS

a) Histochemical ß-glucuronidase (GUS) assays

Histochemical GUS assays were performed on the leaves of putative genetically modified Eucalyptus clones and seedling-derived material as described by Draper et al. (1988). Leaf explants were transferred to a petri dish containing fixation solution (100 ml double distilled water containing 750 µl 40% formaldehyde, 2 ml 0.5 M MES and 5.46 g $l^{-1}$ Mannitol). The petri dish was placed in a vacuum desiccator and the vessel was evacuated several times until all of the explants were submerged in the fixation solution. The explants were incubated for 45 minutes at room temperature and then washed twice in 50mM sodium phosphate buffer (pH 7.0). The explants were then transferred into a 2mM 5-bromo-4-chloro-3-indoyl glucuronide (X-GLUC) solution made up in 50mM sodium phosphate buffer (pH 7.0). The X-GLUC solution was vacuum infiltrated into the explants several times, the dish sealed with Nescofilm and then incubated at 37°C overnight. The reaction was stopped by transferring the explants to 70% ethanol. GUS activity could be detected by the presence of an insoluble blue stain.

b) Detection of genes transfered to transgenic Eucalyptus plants by Southern blotting and hybridisation.

DNA extraction was carried out as described by Keil and Griffin (1994). 10 micrograms of DNA isolated from transformed Eucalyptus plants were digested with with Kpn1 and Xba1 in the appropriate restriction buffers. To aid the digestion of DNA, casein was added to the

restriction mixture at a final concentration of 0.1 mg/ml (Drayer and Schulte-Holthausen, 1991). The restrictions were carried out at 37°C overnight. Electrophoresis of the samples, Southern blotting and hybridisation were performed as described by Sambrook et al. (1989). The plasmid pJIT65 (Guerineau, 1990) was digested with Eco RV and the plasmid pCaMV digested with Bam H1. The resulting restriction fragments were separated by electrophoresis on a 1.5% agarose gel (Sambrook et al., 1989). A 2kb (approximately) DNA fragment containing part of the coding sequence of the GUS gene and the Cauliflower Mosaic Virus 35S gene terminator region and a 1.0 kb (approximately) DNA fragment containing the NPT2 coding sequence were eluted from the gel by the method of Heery et al. (1990). The eluted fragments were radiolabelled by the method of Feinberg and Vogelstein (1983), using the random primer labelling kit supplied by Boehringer Manheim and used as hybridisation probes.

c) Results

The process of the invention as described in the Examples set out above enabled transformed Eucalyptus plants to be produced efficiently and in short periods of time, even from explants originating from mature plants (clones) which had previously been grown in the field and for which production of transformed plants has not proved possible. The efficiency of these methods enabled large populations of plants each resulting from individual transformation events to be produced from any one of the Eucalyptus species or hybrids transformed. In all of the examples, genetically modified shoots were obtained via organogenesis from genetically modified callus. In some cases, mixed organogenesis and somatic embryogenisis could be observed in some of the cultures, particulary

if culture periods on regeneration media were continued for extended periods. In all of the methods described, viable plants were recovered that exhibited normal phenotypes when grown greenhouse conditions. A high proportion (in excess of 70%) of the genetically modified plants from any one of the Eucalyptus species or hybrid transformed were found to express the ß-glucuronidase gene as determined by histochemical staining. Similarly, at least 80% of the regenerated shoots were found to contain at least one of the genes from the T-DNA of pSCV1.6 integrated into the genome of the Eucalyptus species or hybrid.

REFERENCES

Beck E; Ludwig G; Auerswald E A; Reiss B; Schaller H: (1982). Gene 19, 327-336.

Bourgin JP; Nitsch JP: (1967). Ann. Physiol. Veg. 9, 377-382.

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988). Plant genetic transformation **and** gene expression. A laboratory manual. Blackwell Scientific Publications, Oxford.

Dreyer, K. and Schulte-Holthausen, H. (1991). Nucl. Acids Res., 19, 4295.

Edwards G A. (1988): Ph. D. Thesis. University of Durham.

Feinberg AP; Vogelstein B: (1983). Anal. Biochem. 132, p6-13.

Fromm M; Taylor L P; Walbot V: (1986). Nature 319, 791-793.

Guerineau, J.F. (1990). In: pJIT Plasmid Directory, Institute of Plant Science Research, John Innes Institute, Colney Lane, Norwich.

Heery, D.M., Gannon, F., and Powell, R. (1990). Trends Genet., 6, 173.

Holsters B; Villaroel R; Gielen J; Seurinck J; De Greve H; Van Montagu M; Schell J: (1983). Mol. Gen. Genet. 190, 35-41.

Hood E E; Helmer G L; Frayley R T; Chiton M-D: (1986). J. Bacteriol. 168, 1291-1301.

Keil M; Griffin AR: (1994). Theor Appl Genet 89, 442-450.

Murashige T. and Skoog F: (1962). Phys. Plant. 15, 473-497.

Morel and Wetmore: (1951). Am. J Bot. 38, 141-143.

Nitsch JP; Nitsch C: (1965). Ann. Physiol. Veg. 7, 251-256.

Olszewski N E; Martin F B; Ausubel F M: (1988). Nucleic Acids Research 16, 10765-10782.

Peralta E G; Hellmiss R; Ream W: (1986). EMBO J. 5, 1137- 1142.

Sambrook J; Fritsch E F; Maniatis T: (1989). Molecular cloning: A laboratory manual. Second Edn. Cold Spring Harbour Press.

Simon R; Priefer U; Puhler A: (1983). Biotechnology 1, 784-791.

Simpson R B; O'Hara P J; Kwok W; Montoya A L; Lichenstein C; Gordon M P; Nester E W: (1982). Cell 29, 1005-1014.

Thomas C M; Meyer R; Helsinki D R: (1980). J. Bacteriology 141, 213-222

Valantine C R I; Kado C I: (1989). In: Promiscuous plasmids of the Gram-negative bacteria. C. M. Thomas (Ed). Academic Press. p125-163.

Vancanneyt G; Schmidt R; O'Connor-Sanchez A; Willmitzer L; Rocha- Sosa M: (1990). Mol. Gen. Genet., 220, 245-250.

Wen-jun S; Forde B G. (1989). Nucleic Acids Res. 17, 8385.

Yannish-Perron C; Vieira J; Messing J: (1985). Gene 33, 103-119.

Yenofsky R L; Fine M; Pellow J W: (1990). Proc. Natl Acad. Sci. USA. 87, 3435-3439.

C L A I M S

1. A process for producing genetically modified Eucalyptus plant material comprising one or more stably incorporated DNA sequences of interest, which process comprises subjecting Eucalyptus cells or tissue to Agrobacterium mediated transfer of the DNA sequence(s) of interest, inducing shoot formation in transformed cells or tissue, and selecting transformed material, the induction of shoot formation being carried out in the presence of N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea.

2. A process as claimed in claim 1, wherein selection of transformed shoots is carried out using geneticin (G-418) or neomycin as selective agent.

3. A process as claimed in claim 1 or claim 2, wherein the tissue or cells is/are clonal tissue or cells derived vegetatively, directly or indirectly, from vegetative tissue of a mature tree of E. grandis or E. dunnii, or of a variety, cultivar or hybrid thereof.

4. A process as claimed in claim 1 or claim 2, wherein the tissue or cells is/are derived vegetatively, directly or indirectly, from vegetative tissue of a mature tree of E. saligna or E. camaldulensis, or of a variety, cultivar or hybrid thereof.

5. A process as claimed in claim 3 or claim 4, wherein the tissue comprises petioles, leaves or stems.

6. A process as claimed in any one of claims 1 to 4, wherein the tissue or cells is/are derived from seedlings of E. globulus, E. nitens or E. dunnii.

7. A process as claimed in any one of claims 1 to 6, wherein induction of shoot formation and selection of transformed shoots is carried out by culture on a solid

medium or using another static culture system.

8. A process as claimed in any one of claims 1 to 7, wherein at least one of the DNA sequences is capable of imparting a phenotypic property to the Eucalyptus.

9. A process for producing a genetically modified Eucalyptus plant, which comprises growing genetically modified plant material obtained according to a process as claimed in any one of claims 1 to 8 into a plant, optionally after vegetative propagation (cloning) of the genetically modified plant material.

10. A process for producing a cloned Eucalyptus plant, which comprises vegetatively propagating (cloning) a genetically modified Eucalyptus plant that has been obtained by growing genetically modified plant material obtained according to a process as claimed in any one of claims 1 to 8 into a plant, the genetically modified plant material optionally being vegetatively propagated (cloned) before it is grown into a plant.

11. A mature adult Eucalyptus tree obtained by growing a Eucalyptus plant obtained by a process as claimed in claim 9 or claim 10.

12. Timber, pulp or fuel wood obtained from a mature adult Eucalyptus tree as claimed in claim 11.

1/2

FIG.1

MAP of pSCV1

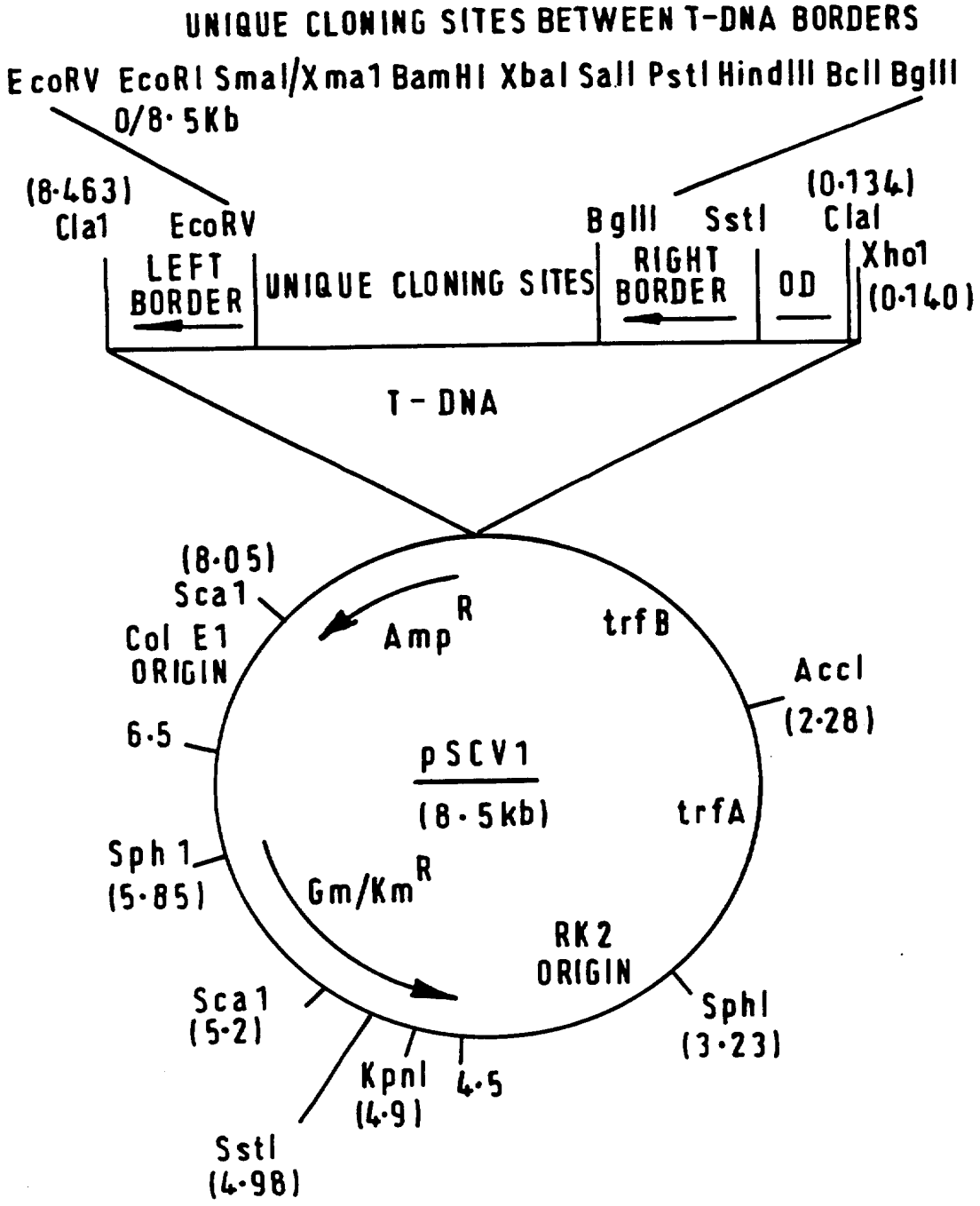
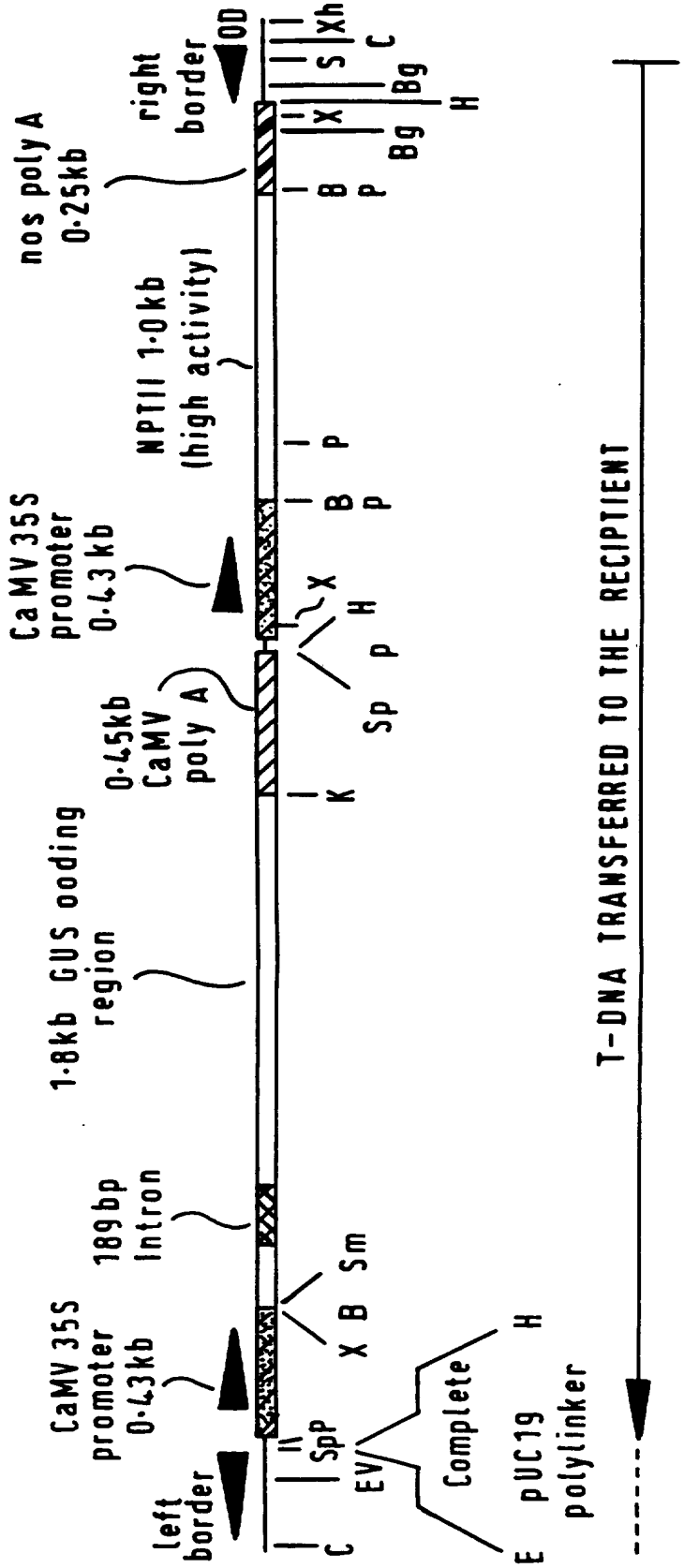


FIG. 2

T-DNA of pSCV1-6



# INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 96/00712

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H4/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | DATABASE WPI<br>Section Ch, Week 9049<br>Derwent Publications Ltd., London, GB;<br>Class A97, AN 90-366268<br>XP002005320<br>& JP,A,02 265 419 (OJI PAPER KK) , 30 October 1990<br>see abstract<br>--- | 1-12 |
| Y | GB,A,2 211 204 (OJI PAPER CO) 28 June 1989<br>see page 2, line 31 - line 33<br>see page 3, line 26 - line 28<br>--- | 1-12 |
| Y | WO,A,91 18094 (AGRACETUS ;WISCONSIN ALUMNI RES FOUND (US)) 28 November 1991<br>see the whole document<br>---<br>-/-- | 1-12 |

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| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | MEETING OF THE BOTANICAL SOCIETY OF FRANCE ON THE MOLECULAR AND CELLULAR BASES OF MORPHOGENESIS IN PLANTS, PARIS, FRANCE, DECEMBER 5, 1992. ACTA BOTANICA GALLICA 140 (6). 1993 (1994). 702., XP000573448 AZMI A ET AL: "Comparative efficacy of different strategies of transformation-regeneration in Eucalyptus globulus." see the whole document | 1-12 |
| A | PLANT CELL REPORTS, vol. 9, 1991, pages 590-594, XP000574124 MCCOWN, B.H., ET AL.: "Stable transformation of Populus and incorporation of pest resistance by electric discharge particle acceleration" see page 592, right-hand column, line 24 - line 31 | 1-12 |
| A | PLANT CELL REPORTS 13 (8). 1994. 473-476., XP000573015 LAINE E., ET AL.: "Regeneration of plants from leaf explants of micropropagated clonal Eucalyptus grandis." cited in the application see the whole document | 1-12 |
| A | US,A,5 240 839 (SERRES RODNEY A ET AL) 31 August 1993 see column 4, line 59 - line 68 | 1-12 |
| A | DATABASE WPI Section Ch, Week 8715 Derwent Publications Ltd., London, GB; Class C03, AN 87-106306 XP002005321 & JP,A,62 055 020 (OJI PAPER KK) , 10 March 1987 see abstract | 1-12 |
| A | CHEMICAL ABSTRACTS, vol. 117, no. 1, 6 July 1992 Columbus, Ohio, US; abstract no. 4434, OTA, TSUTOMU ET AL: "Genetically-engineered woody plants" XP002005318 see abstract & JP,A,04 053 429 (OJI SEISHI K. K., JAPAN) | 1-12 |

-/--

2

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/00712

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DATABASE WPI<br>Section Ch, Week 9425<br>Derwent Publications Ltd., London, GB;<br>Class C07, AN 94-204914<br>XP002005322<br>& JP,A,06 141 719 (OJI PAPER CO) , 24 May 1994<br>see abstract | 1-12 |
| A | CHEMICAL ABSTRACTS, vol. 121, no. 23,<br>5 December 1994<br>Columbus, Ohio, US;<br>abstract no. 273846,<br>HIBINO, TAKASHI ET AL: "Preparation of transgenic broadleaf trees with reduced cinnamyl alcohol dehydrogenase activity"<br>XP002005319<br>see abstract<br>& JP,A,06 181 775 (MITSUI GYOSAI SHOKUBUTSU BAIO, JAPAN) | 1-12 |
| A | EP,A,0 227 264 (CALGENE INC) 1 July 1987<br>see column 2, line 40 - line 53 | 1-12 |
| A | EP,A,0 519 758 (UNIV GUELPH) 23 December 1992<br>see page 6, line 28 - line 38<br>see page 11, line 20 - line 31 | 1-12 |

2

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 96/00712

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| GB-A-2211204 | 28-06-89 | FR-A- | 2621780 | 21-04-89 |
| | | JP-A- | 2138966 | 28-05-90 |
| WO-A-9118094 | 28-11-91 | CA-A- | 2064017 | 25-11-91 |
| US-A-5240839 | 31-08-93 | NONE | | |
| EP-A-0227264 | 01-07-87 | US-A- | 4795855 | 03-01-89 |
| | | AU-B- | 597916 | 14-06-90 |
| | | AU-B- | 6512286 | 21-05-87 |
| EP-A-0519758 | 23-12-92 | CA-A- | 2071767 | 21-12-92 |
| | | US-A- | 5477000 | 19-12-95 |